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Research Article

FRACTIONATION OF THE *N*-HEXANE EXTRACT OF *GARCINIA BANCANA* MIQ. (MANGGIS HUTAN) LEAVES AND ITS ANTIOXIDANT ACTIVITY BASED ON 1,1-DIPHENYL-2-PICRYLHYDRAZYL AND FERRIC REDUCING ANTIOXIDANT POWER ASSAYS

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ABSTRACT

Objective: To assess the antioxidant activity from another part of the plant, in this study, leaf extracts in *n*-hexane were fractionated.

Methods: Ten fractions were obtained and tested *in vitro* for antioxidant activity using two methods, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP), to identify the most active fraction.

Results: The IC_{50} of the most active fraction was 36.24 µg/mL using the DPPH method, and the EC_{50} was 39.54 µg/mL using the FRAP method. The most active fraction was also shown to contain terpenoids.

Conclusion: The most active fraction of an n-hexane extract of the leaves of *Gacinia bancana* Miq., which was tested by both DPPH and FRAP methods had antioxidant activities with IC_{50} and EC_{50} values of 36.2482 µg/mL and 39.5442 µg/mL, respectively. Phytochemical screening showed that active fraction contains terpenoids.

Keywords: 1,1-diphenyl-2-picrilhydrazyl, Antioxidant, Ferric reducing antioxidant power, *Gacinia bancana* Miq.

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INTRODUCTION

Free radicals are molecules containing one or more unpaired electrons in their outermost molecular orbitals. Excess free radicals in the body can lead to degenerative diseases such as stroke, cancer, and heart attack [1]. To overcome the problem of free radicals, the body requires antioxidants that can scavenge free radical compounds and thus prevent the damage caused by the chain reaction of free radical formation elicited by oxidative stress [2]. Antioxidants are basically divided into two types: Natural and synthetic. Commonly used synthetic antioxidants include butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, and tertiary butyl hydroquinone, which are all carcinogenic and hence harmful [3-5]. Hence, the use of natural antioxidants is considered more suitable with respect to human health.

Some species of *Garcinia* have strong antioxidant activity and are a source of natural antioxidants. Methanol, ethyl acetate, and n-hexane extracts of *Garcinia kydia* Roxb. leaves exhibited EC_{50} values on ferric reducing antioxidant power (FRAP) assay of 18.4, 12.4, and 31.3 µg/mL, respectively [6]. Similarly, methanol, ethyl acetate, and n-hexane extracts of *Gacinia bancana* Miq. leaves exhibited antioxidant activity with IC_{50} values of 26.9, 51.3, and 19.9 µg/mL [7].Another with an n-hexane extract of *G. bancana* Miq stem bark tested in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) produced an IC_{50} value of 17 µg/mL and identified the presence of bancanon, a triterpenoid, that is suspected to have antioxidant potential [5]. Other compounds found in many species of *Garcinia,* namely xanthone, benzophenone, and triterpenoid, all possess antibiotic, anticancer, and antioxidant activities [7].

This previous research indicates that the IC_{50} of n-hexane extracts of *G. bancana* Miq. is smaller than that of ethyl acetate and methanol extracts. Therefore, in this study, n-hexane *G. bancana* Miq. leaf extracts were fractionated using column chromatography to obtain the most active antioxidant by *in vitro* screening using the DPPH and FRAP methods. Phytochemical screening of the most active fraction was then carried out to identify the active ingredient.

METHODS

Working procedures included material preparation, fractionation, and antioxidant activity testing by determining IC_{50} using DPPH and FRAP assays to identify the active fraction and determination of the chemical content of the active fraction of an n-hexane extract of *G. bancana* Miq. leaves by phytochemical screening.

RESULTS AND DISCUSSION

Chromatography

Column chromatography produced 310 fractions that were evaporated, subjected to thin-layer chromatography (TLC) on a silica gel plate, stained from KLT, and examined under ultraviolet (UV) light. Fractions containing the same spots were combined into the same fraction, and all fractions were then dried and weighed before testing. The weights of the resulting ten fractions are shown in Table 1.

Preliminary testing with DPPH spray

The ten samples were run on a F_{254} silica gel plate and then sprayed with a 100 µg/mL DPPH solution. Positive antioxidant results were indicated by the formation of a pale-yellow circle with a purple background [8].

Of the ten fractions, no antioxidant activity was found in fractions 1, 2, 3, 4, and 5, while the 6th fraction produced very small yellow spot. The highest antioxidant activity was found in the 7th and 8th fractions, while the yellow color faded and shrunk again in the 9th and 10th fractions. These results, however, were not conclusive and further testing was required.

DPPH antioxidant activity testing

Antioxidant activity was first tested using quercetin as a reference standard at five different final concentrations of 0.25, 0.5, 1, 1.5, and 2 µg/mL and by measurement of absorbance at 517 nm. The determined IC_{50} value was 1.262 µg/mL, and the calibration curve is shown in Fig. 1.

Next, fractions 7, 8, and 9, which had been preliminarily tested qualitatively and exhibited antioxidant activity, were tested more extensively using DPPH. In this spectrophotometric assay, smaller absorption indicates higher antioxidant activity.

Fraction 7 exhibited the smallest absorption and therefore the highest percentage of inhibition value of 28.03% among the three fractions tested, thereby showing the greatest antioxidant activity. The data for the three fractions are presented in Table 2.

Fraction 7 was then analyzed to determine its IC_{50} fraction 7 using different concentrations, namely 72.03, 102.9, 123.48, 154.35, and 185.22 µg/mL, with final concentration based on the dilution factors at the cuvette as 18.0, 25.73, 30.87, 38.59, and 46.31 µg/mL, respectively. This showed that most active fraction of the n-hexane extract *G. bancana* Miq. had an IC_{50} of 36.25 µg/mL (Table 3).

FRAP antioxidant activity testing

Antioxidant testing was repeated using the FRAP method to confirm the DPPH results. In contrast to the DPPH method, in the FRAP method, the samples with higher antioxidant also have the highest absorbance [9].

Quercetin was again used as a reference standard, with final concentrations of 1, 2, 3, 4, and 5 µg/mL producing an EC_{50} value of 2.61 µg/mL (Fig. 2).

The three fractions previously tested with DPPH were prepared at the same concentration of 100 µg/mL, and uptake was measured using a UV-visible spectrophotometer at the optimum wavelength of 594 nm. The concentration data and percentage of sample capacity are shown in Table 4.

The most active fraction was tested by using concentration 25.73, 36.02, 51.45, 67.91, and 77.18 µg/mL. The resulting data (Table 5) were used to generate an inhibition curve (Fig. 3). This showed that the n-hexane leaf extract of *G. bancana* Miq. had an EC_{50} of 39.54 µg/mL when measured by the FRAP method.

Phytochemical screening

Phytochemical screening was carried out qualitatively using TLC, and the test results are shown in Table 6.

Flavonoid test

The flavonoid test used quercetin as a standard, and the plate was examined under 366 nm UV light. Red streaks and spots were observed in the sample that was different from the standard (Fig. 4).

Table 1: Fraction data result

Fraction	Weight extract (g)
1	0.0131
2	4.6844
3	0.8445
4	2.0098
5	0.3084
6	0.9247
7	5.3835
8	1.3532
9	0.3267
10	7.0573

Table 2: Concentrations and %DPPH inhibition data for sample fractions

Fraction	Final concentration (µg/mL)	% inhibition
7	25.725	28.03
8	27.125	26.19
9	27.25	3.45

DPPH: 1,1-diphenyl-2-picrylhydrazyl

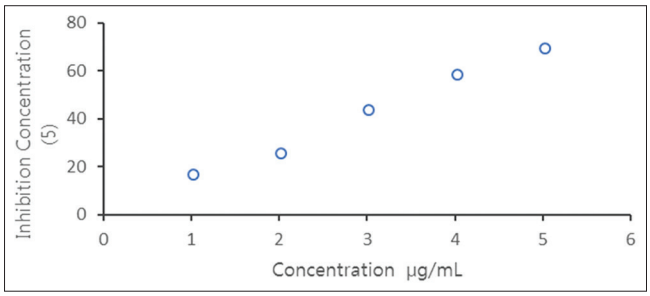

Fig. 1: Quercetin antioxidant activity curve using the 1,1-diphenyl-2-picrylhydrazyl method

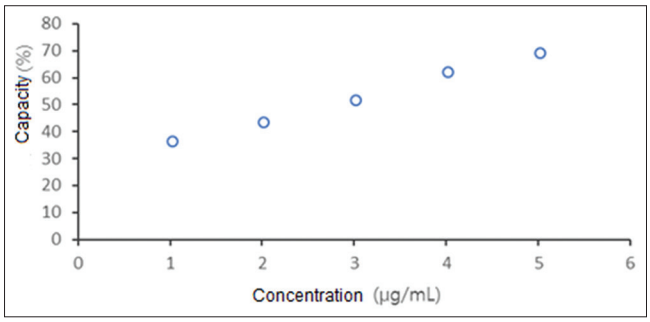

Fig. 2: Quercetin antioxidant activity curve by the ferric reducing antioxidant power method

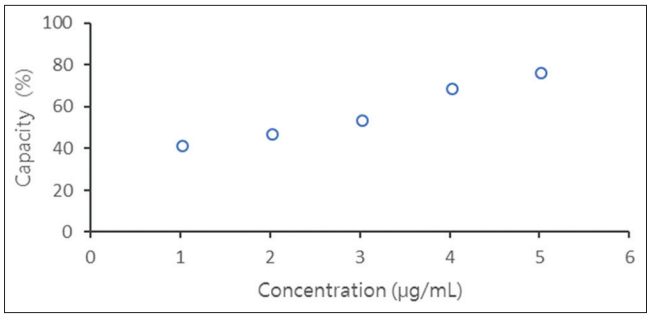

Fig. 3: Most active fraction antioxidant activity curve using the ferric reducing antioxidant power method

Table 3: %DPPH inhibitory data and IC_{50} of the most active fraction

Final concentration (µg/mL)	Inhibition (%)	Linear regression equation	IC_{50} (µg/mL)
18.00	34.05	y=0.877x+18.2	36.25
25.72	39.43		
30.87	46.05		
38.58	53.84		
46.30	57.52		

DPPH: 1,1-diphenyl-2-picrylhydrazyl

Alkaloid test
Tetrandrine is used as the standard in the alkaloid test. Spots should become visible after being sprayed by Dragendorff reagent, with positive results appearing as yellow spots (Fig. 5).

Terpenoid test
β-sitosterol was used as a standard for terpenoids The plate was run in a hexane and ethyl acetate solvent (9:1) and then sprayed with vanillin and 10% H_2SO4 in ethanol and heated (Fig. 6).

The study began with separation using column chromatography to produce ten fractions of an n-hexane leaf extract of *G. bancana* Miq (Table 1). A preliminary test with a DPPH spray was conducted to determine which fractions contained potential antioxidant activity. At this stage, the results were not conclusive because the spray reaction is only a supporting reaction and further quantitative testing was required in the form of standard antioxidant activity tests.

Quercetin is used as a reference standard in these tests because it is a powerful antioxidant that can protect body tissues from oxidative stress [5,10]. The quercetin IC_{50} determined using DPPH was 1.262 µg/mL, which was in good agreement with a previous study that reported an IC_{50} value for quercetin of 1.565 µg/mL [8]. Based on previous research, DPPH measurements using UV-visible spectrophotometer also obtained maximum absorption at 517 nm [8,11-13].

The test conducted on the three fractions that exhibited the highest activity in the DPPH spray test showed that fraction 7 exhibited the highest activity and its IC_{50} was subsequently determined to be 36.248 µg/mL. Furthermore, with decreasing absorption of DPPH, the radical capture activity increased [14]. In a previous study into the antioxidant activity of the same plant species, and using the same extraction solvent (n-hexane) except on *G. bancana* Miq stem bark, the DPPH method produced an IC_{50} of 17.78 µg/mL. The difference in IC_{50} values may be due to differences in the plant parts tested or in the equipment and materials used for testing. Nevertheless, the IC_{50} values obtained still exhibited very strong antioxidant activity because they were below 50 µg/mL [15].

More than one method is used to test the antioxidant capacity of the extract fraction due to the complex nature of phytochemical compounds [16]. The FRAP method was used to supplement and reinforce the results obtained using the DPPH method.

FRAP testing also used quercetin as a reference standard and a UV-visible spectrophotometer set to an optimum wavelength of 594 nm, in contrast to previous research, which used 593 nm [17,18,19]. This may be due to differences in the equipment used. The quercetin EC_{50} obtained was 2.61 µg/mL, which was consistent with previous *in vitro* research reporting a value of 2.4 µg/mL [20].

In contrast to the DPPH method, the FRAP method showed good antioxidant activity for samples with highest absorbance [9]. FRAP testing also showed that fraction 7 had the highest antioxidant activity, in agreement with the DPPH method. Thus, it is likely that fraction 7 had the best antioxidant activity, and hence, its EC_{50} was determined.

The FRAP method is the only method that measures antioxidants and reductants in a sample directly, while other methods measure antioxidant activity indirectly by measuring free radical inhibition produced in the solution and the results depend on the reactive species being used [18, 21-23]. Therefore, the value of the result of the reduction in FRAP is determined by the value of EC_{50} (Fig. 2 and Table 5).

Table 4: Concentration and percentage sample capacity data

Fraction	Final concentration (µg/mL)	Capacity percentage (%)
7	51.45	54.53
8	54.25	50.99
9	54.5	51.53

FRAP testing produces an EC_{50} of 39.54 µg/mL. Prior research on the antioxidant activity of the *Garcinia* genus using the FRAP method showed that an n-hexane leaf extract of *G. kydia* Roxb. had an EC_{50} of 31.26 µg/mL, which was greater than that of ethyl acetate and methanol extracts, which had EC_{50} values of 12.389 and 18.448 µg/mL, respectively [6]. This is because the *Garcinia* species differed from the one used in this study, so the compounds contained in the extracts may have been different.

Possible components of the most active fraction (Fraction 7) were identified by TLC. Flavonoid testing used quercetin as a standard because quercetin is a flavonol [10]. After elution, the spots are not clearly visible and hence need spraying with $AlCl_3$ for viewing under a 366 nm UV light bulb [24]. The sample contained red streaks and spots that were different from the standard (Fig. 4). This is likely because the sample was derived from a chlorophyll-containing leaf extract so and so chlorophyll, which can interfere with the chlorophyll-red color-chlorophyll color zone, may have comigrated and covers flavonoid compounds in the sample. Further tests on the flavonoid content of n-hexane extract of *G. bancana* Miq are required.

Tetrandrine was used as the standard in the alkaloid test, but none were found (Fig. 5). β-sitosterol was used as the standard in the terpenoid

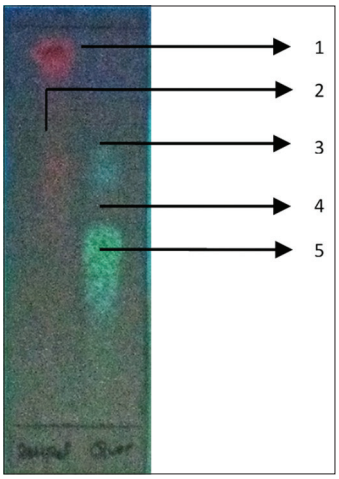

Fig. 4: The most active chromatography fraction of an n-hexane leaf extract of *G. bancana* Miq. and quercetin using a chloroform:acetone:formic acid (8:1:1) mobile phase and after spraying $AlCl_3$ 5% on 366 nm ultraviolet light

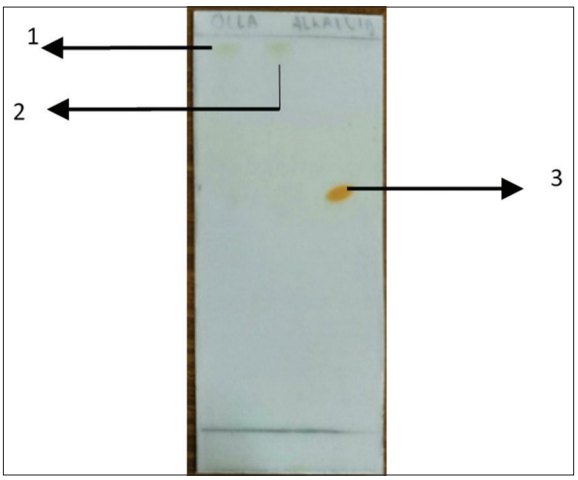

Fig. 5: The most active chromatography of an n-hexane leaf extract of *Garcinia bancana* Miq. (1). (2) and tetrandrine (3) with toluene toxic phase:ethyl acetate:methanol:ammonia (10:9:6:0.3) after spraying Dragendorff reagents

Table 5: FRAP % capacity and value of EC_{50} most active fraction

Final concentration (µg/mL)	Capacity percentage (%)	Linear regression equation	EC_{50} (µg/mL)
25.725	42.14	0.6823x+23.019	39.5442
36.015	47.79		
51.45	54.53		
67.914	69.68		
77.175	77.16		

FRAP: Ferric reducing antioxidant power

Table 6: Results of phytochemical screening with TLC plates

Compound test	Standard	Mobile phase	Spray	Result
Flavonoid	Quercetin	Chloroform:acetone:formic acid (8:1:1)	$AlCl_3$	-
Alkaloid	Boldine	Toluene:ethyl acetate: methanol:ammonia (10:9:6:0.3)	Dragendorff	-
Terpenoid	β-sitosterol	n-Hexane: ethyl acetate (9:1)	H_2SO_4-Vanillin	+

TLC: Thin-layer chromatography

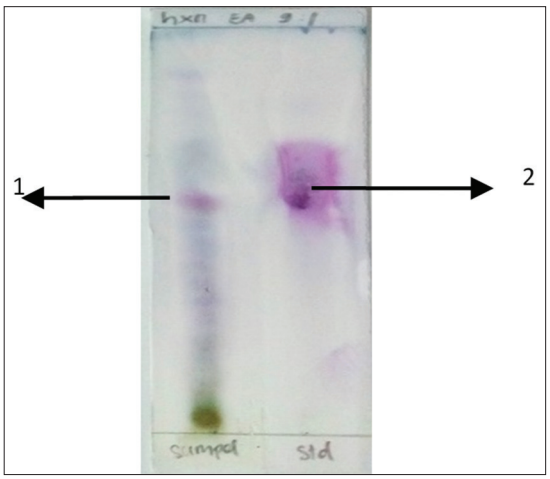

Fig. 6: The most active chromatography fraction of an n-hexane leaf extract of *Garcinia bancana* Miq. (1) and β-sitosterol (2) using a hexane and ethyl acetate (9:1) mobile phase and after spraying with 1% vanillin and 10% H_2SO_4 and heating

test. The sample produced a chromatogram and color pattern similar to the standard, indicating that terpenoid group compounds were present (Fig. 6).

CONCLUSION

Antioxidant testing was repeated using the FRAP method to confirm the DPPH results. In the FRAP method, the samples with higher antioxidant also have the highest absorbance. Furthermore, the most active fraction of an n-hexane extract of the leaves of *G. bancana* Miq., which was tested by both DPPH and FRAP methods, had antioxidant activities with IC_{50} and EC_{50} values of 36.2482 µg/mL and 39.5442 µg/mL, respectively. Phytochemical screening showed that the active fraction contains terpenoids.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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